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Lentiviral Vector-Based Prime/Boost Vaccination against AIDS: Pilot Study Shows Protection against Simian Immunodeficiency Virus SIVmac251 Challenge in Macaques▽

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AIDS vaccination has a pressing need for more potent vaccination vectors capable of eliciting strong, diversified, and long-lasting cellular immune responses against human immunodeficiency virus (HIV). Lentiviral vectors have demonstrated efficiency not only as gene delivery vehicles for gene therapy applications but also as vaccination tools. This is likely due to their ability to transduce nondividing cells, including dendritic cells, enabling sustained endogenous antigen presentation and thus the induction of high proportions of specific cytotoxic T cells and long-lasting memory T cells. We show in a first proof-of-concept pilot study that a prime/boost vaccination strategy using lentiviral vectors pseudotyped with a glycoprotein G from two non-cross-reactive vesicular stomatitis virus serotypes elicited robust and broad cellular immune responses against the vector-encoded antigen, simian immunodeficiency virus (SIV) GAG, in cynomolgus macaques. Vaccination conferred strong protection against a massive intrarectal challenge with SIVmac251, as evidenced both by the reduction of viremia at the peak of acute infection (a mean of over 2 $\log_{10}$ fold reduction) and by the full preservation of the $CD28^+$ $CD95^+$ memory $CD4^+$ T cells during the acute phase, a strong correlate of protection against pathogenesis. Although vaccinees continued to display lower viremia than control macaques during the early chronic phase, these differences were not statistically significant by day 50 postchallenge. A not-optimized SIV GAG antigen was chosen to show the strong potential of the lentiviral vector system for vaccination. Given that a stronger protection can be anticipated from a modern HIV-1 antigen design, gene transfer vectors derived from HIV-1 appear as promising candidates for vaccination against HIV-1 infection.

The AIDS vaccination field is currently facing a drawback after the failure of the phase IIb test-of-concept STEP trial of the MERCK's adenovirus serotype 5 (Ad5)-based vaccine candidate (8, 36, 52). There was no protection induced by the MRKAd5 human immunodeficiency virus type 1 (HIV-1) GAG/POL/NEF vaccine and no reduction of viremia in cases of infection, and it appears that HIV-1 incidence was even higher in vaccinated men with preexisting immunity to Ad5 (8, 36). There is therefore a pressing need to design and test new strategies, including new viral vectors. Many studies have highlighted the critical role played by $CD8^+$ T cells in controlling HIV infection (12, 27, 29, 41, 48, 50, 53) and suggested that an effective vaccine should induce vigorous, broad, and long-lasting $CD8^+$ T-cell responses (2, 5). However, no clear-cut immune correlates of protection have been described thus far, and yet several viral vectors shown to elicit specific SIV $CD8^+$ T-cell responses have subsequently failed to control viremia in SIV/macaques models (reviewed by Schoenly and Weiner [51]).

One unique feature of HIV-1 and lentiviruses biology is their ability to infect nondividing cells through active nuclear import of their genome into the nucleus of the infected cell (59). This precious property was exploited for the design of lentivirus-derived retroviral vectors, which in contrast to classical Moloney retroviral vectors, efficiently transduce nondividing target cells, such as adult and embryonic stem cells, neurons, or hepatocytes. Lentiviral vectors constitute a major technological breakthrough in the field of gene transfer. Notably, a number of successful gene therapy preclinical studies have translated into an increasing number of approved or ongoing human clinical trials worldwide (http://www.wiley.co.uk/genmed/clinical/) including, among them, several in the AIDS field.

When applied as vaccination tools, lentiviral vectors efficiently transduce dendritic cells (DC), enabling stable delivery of the antigen and thus sustained presentation by DC of the encoded antigen through the endogenous pathway (22). Lentiviral vector-mediated antigen presentation will persist for the whole in vivo lifetime of the DC, i.e., until they are eliminated by the innate (7) and adaptive immunity they have induced. This sustained endogenous antigen presentation probably accounts for the potent induction of high proportions of specific cytotoxic T cells and long-lasting memory T cells that we and others have reported after direct injection of lentiviral vector particles in various mice models (6, 9, 10, 21). It appears, in several comparative studies (14, 17, 22, 47, 58) that lentiviral vectors are more potent to elicit cellular protective immunity

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TABLE 1. Immunization groups and experimental design[a]

Treatment group	No. of animals	Vaccine	Subgroup	Animals	Prime particles[b]	Boost particles[c]	SIVmac251 challenge (AID_{50})[d]
Vaccines	6	TRIP-SIVmac239 GAG	Low dose	20022, 20089	2.5×10^7 TU	1.2×10^8 TU	500
			Medium dose	20293, 20056	1×10^8 TU	1.2×10^8 TU	500
			High dose	20195, 20158	2.5×10^8 TU	1.2×10^8 TU	500
GFP control	2	TRIP-GFP		21544, 20456	6,863 ng of p24	6,018 ng of p24	500
Naive	4	None		15661, 14184, 15885, 14468	None	None	500

[a] Twelve outbred males and adult cynomolgus macaques (*Macaca fascicularis*) from the Indian Ocean Island of Mauritius were included in the preclinical trial. They were negative for SIV, herpesvirus B, filovirus, STLV-1, SRV-1, SRV-2, measles virus, hepatitis B-HbsAg, and hepatitis B-HBcAb before inclusion in the study. Immunizations, blood collections, and challenges were handled in accordance with EU guidelines for experiments using nonhuman primates (document 2001-486) and approved by the Institut Pasteur review board. Immunizations were done by subcutaneous injections on days 0 and 79 of lentiviral particles pseudotyped with two different envelopes: the glycoproteins G from two non-cross-reactive VSV serotypes: Indiana and New Jersey. The doses of lentiviral vector particles were expressed as TU/animal and as ng of p24/animal. Six animals were immunized with three doses (low, medium, and high) of lentiviral vectors encoding a nonsecreted form of SIVmac239 GAG (myristoylation deficient, Δ*myr*). Because of the absence of a dose response after the first injection, all six vaccinated animals received the same medium dose of vector for the second injection. Two control animals were immunized with lentiviral vector encoding an irrelevant antigen, GFP, at the same p24 dose as the high-dose relevant subgroup. Vaccinated, GFP control, and unvaccinated macaques were challenged intrarectally at 76 days postboost with a high dose of pathogenic SIVmac251 (500 AID_{50}) compared to 20 to 50 AID_{50} usually used in studies of the protective efficacy of vaccines. The virus stock was from A.-M. Aubertin, Université Louis Pasteur, Strasbourg, France. The GAG from SIVmac251 used for the challenge closely matches the GAG from SIVmac239 encoded by the vaccine (homologous challenge).

[b] Pseudotyped with VSV-G Indiana at day 0.

[c] Pseudotyped with VSV-G New Jersey at day 79 postprime.

[d] At day 76 postboost.

than other viral vectors (including adenoviral and pox vectors) or other vaccine strategies (such as DNA vaccine and peptide-pulsed or mRNA electroporated DC).

We assessed here whether HIV-1-derived lentiviral vectors could confer protective cellular immunity against simian immunodeficiency virus (SIV) infection and simian AIDS. We opted for the model of SIVmac251 infection of cynomolgus macaques, which displays viral load levels and a variety of progression rates similar to those seen in HIV-1 infection in humans (3, 28, 44, 54). We have recently shown that, unlike T cells or macrophages, nonhuman primate DC lack TRIM5α-mediated restriction and are equally transduced by HIV-1-derived lentiviral vector such as human DC, allowing the use of Old World primates as vaccine models to test HIV-1-derived lentiviral vectors (1). We show that a prime/boost vaccination regimen using lentiviral vector encoding a nonoptimized SIV GAG antigen induced specific T-cell responses capable of controlling the early viral replication by reducing plasma viral loads at the peak of acute infection by >200-fold and by fully preventing the acute $CD28^+$ $CD95^+$ memory $CD4^+$ T-cell depletion.

MATERIALS AND METHODS

Vector production and titration. Nonreplicative vector particles were produced by transient calcium phosphate transfection of 293T cells (25) with a vector plasmid containing the HIV-1 *cis*-active elements (long terminal repeat, encapsidation signal Ψ, RRE, and DNA Flap cPPT/CTS) and the transgene under the control of the ieCMV promoter, TRIP-ΔU3-CMV-SIVmac239GAGΔmyr-WPRE (TRIP-SIVmac239 GAG) or TRIP-ΔU3-CMV-eGFP-WPRE (TRIP-GFP), an encapsidation plasmid containing the HIV-1 genes gag, pol, tat, and rev and an envelope expression plasmid encoding the glycoprotein G from vesicular stomatitis virus (VSV) serotype Indiana (pHCMV-G, GenBank no. AJ318514) or New Jersey [pcDNA3.1(−)NJ WPRE]. To construct the pcDNA3.1(−)NJ WPRE, pBS-NJG (GenBank no. V01214) (46) was digested with XhoI and NotI and cloned into the pcDNA3.1(−) vector (Invitrogen). To increase expression, a WPRE (for woodchuck posttranscriptional regulatory element) sequence, preamplified by PCR and cloned into a TOPO TA cloning vector, was added by EcoRI digestion.

Vector HIV-1 p24 contents were measured by enzyme-linked immunosorbent assay (ELISA; Perkin-Elmer Life Sciences), and gene transfer titers were determined by real-time PCR on cell lysates from transduced 293T cells and expressed as transduction unit (TU)/ml or by flow cytometry (24).

Innate cytokines. The presence of alpha interferon (IFN-α; PBL Biomedical Laboratories), interleukin-6 (IL-6; U-Cytech Bioscience), and tumor necrosis factor alpha (TNF-α; U-Cytech Bioscience) in the plasma shortly after subcutaneous injection was measured by ELISA according to the manufacturer's instructions.

IFN-γ ELISPOT. Enzyme-linked immunospot (ELISPOT) assays were performed using MSIP plates (Millipore), the anti-IFN-γ monoclonal antibody GZ-4, and the biotin-conjugated anti-IFN-γ monoclonal antibody 7-B6-1 (Mabtech), streptavidin-alkaline phosphatase and the BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium substrate solution. Spots were numerated by using a Bioreader 4000 (Biosys). Briefly, 0.2×10^6 peripheral blood mononuclear cells (PBMC) per well were restimulated in vitro for 40 h with 11 pools of 5 to 12 overlapping 15-mer peptides (2 μg of each peptide/ml; SIVmac239 GAG [15-mer] Peptides-Complete Set; AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) or with Aldrithiol AT-2-inactivated SIVmac239 (5 μg of total protein/ml) or its control microvesicles (NIBSC, Potters Bar, United Kingdom). The mean number of IFN-γ spot-forming cells (SFC) per million PBMC was calculated from triplicate wells after subtracting the one from control wells (medium without peptide). A pool specific response was considered positive when superior to 200 IFN-γ SFC/million PBMC (three times the median background measured before priming). The cumulative responses shown correspond to the sum of IFN-γ SFC/million PBMC obtained with each pools of peptides. It should be noted that in these settings, SFC may included not only short-lived effector $CD4^+$ and $CD8^+$ T cells but also memory T cells.

Plasma viral loads. Viral RNA was isolated from plasma (200 μl) with TRI Reagent BD (Molecular Research Center). The number of RNA copies was determined in a quantitative one-step reverse transcription-PCR (RT-PCR) using TaqMan EZ RT-PCR (Applied Biosystems) and Mastercycler ep realplex (Eppendorf International). The primers were, respectively, at positions 389 and 456 of the SIVmac251 GAG mRNA genome (forward, TGTCCACCTGCCAT TAAGCCCGA; reverse, GCAGAGGAGGAAATTACCCAGTAC). The TaqMan quantification method was chosen with an internal probe containing the FAM and TAMRA fluorophores, respectively, at the 5′ and 3′ ends (TGTCCA CCTGCCATTAAGCCCGA). The quantity of viral RNA copies was assessed by extrapolation of the threshold fluorescence values onto an internal standard curve prepared from serial dilutions in distilled H_2O of RNA obtained by in vitro transcription with the MAXIscript kit (Ambion) of an SpeI-linearized pGEM-5Zf(+) GAG plasmid. The threshold of detection was 375 viral RNA copies/ml (2.57 $\log_{10}$ RNA copies/ml).

$CD4^+$ T-cell counts. Quantifications of the absolute lymphocyte counts and the proportions of total $CD3^+$ $CD4^+$ $CD8^-$ T cells and $CD28^+$ $CD95^+$ memory $CD4^+$ T cells were described previously (28).

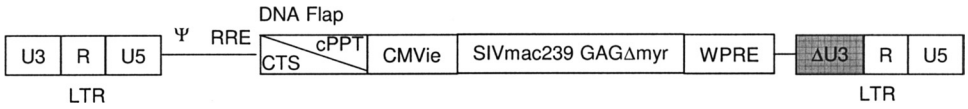


FIG. 1. Structure of the lentiviral vector. A schematic representation of the vector plasmid DNA containing HIV-1 *cis*-active elements used to produce nonreplicative lentiviral vector particles encoding SIVmac239 GAG is shown. LTR, long terminal repeat; cPPT, central polypurine tract; CTS, central termination sequence; ieCMV, human cytomegalovirus immediate-early promoter; ψ, encapsidation signal; RRE, Rev responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

MHC microsatellite haplotype analysis. Genomic DNA were extracted from whole blood collected on EDTA tubes (QIAamp DNA blood; Qiagen). Major histocompatibility complex (MHC) microsatellite haplotype analysis were performed by A. Aarnink and A. Blancher (Laboratoire d'Immunogénétique Moléculaire, Hôpital Rangueil, Toulouse, France) using 20 microsatellite markers spanning the MHC region as described previously and defining seven common haplotypes in Mauritian cynomolgus macaques (19, 57).

Statistics. The nonparametric Mann-Whitney test with two-tailed *P* values and 95% confidence intervals was used to compare viral loads and $CD4^+$ T cells counts between the two groups of vaccinated versus unvaccinated and green fluorescent protein (GFP) control animals. Statistical analyses were computed with GraphPad Prism.

RESULTS

Prime/boost vaccination of cynomolgus macaques with lentiviral vector particles. The study was designed as a small "pilot/test-of-concept" study. Six cynomolgus macaques of Mauritian origin were immunized twice by low-dose subcutaneous injections of HIV-1-derived lentiviral vectors encoding a nonsecreted SIVmac239 GAG protein in its native sequence (TRIP-SIVmac239 GAG). This single and nonoptimized antigen was chosen to highlight the potential of the lentiviral vector system for vaccination. In order to circumvent the presence of neutralizing anti-vector antibodies, and hence to allow an efficient boost effect, a strategy of envelope exchange was designed. Indeed, preparatory experiments in mice had shown that a prime/boost regimen using TRIP-SIVmac239 GAG particles pseudotyped with VSV-G from two non-cross-reactive serotypes, Indiana followed by New Jersey, was more efficient than a homologous prime/boost (data not shown). The structure of the vector, the immunization groups and experimental design are summarized in Table 1 and Fig. 1. The vaccine arm was divided in three subgroups corresponding to three doses of vaccine injected at the prime. For the boost injection, each of the six vaccinated animals was injected with the same dose of vaccine, since there was no clear dose effect at the prime. Extensive preclinical work performed in mice (15, 18, 24, 25) and rats, as well as in vitro transduction efficacy studies of human, macaque, murine, and rat cells, including DC (1, 15, 18), helped us to define a range of doses of vector particles to be tested in monkeys. Vaccinees were compared and contrasted with four naive animals and two animals immunized with a vector encoding an irrelevant antigen, eGFP (GFP control).

Induction of strong immune responses after a single injection. First, it was found that subcutaneous administration of lentiviral particles induced a very mild and transient systemic inflammation, based on IFN-α, IL-6, and TNF-α measured in plasma early after injection (Fig. 2). Second, a single injection of low doses of lentiviral vector was sufficient to induce robust cellular immunity, regardless of the dose received (from 2.5×10^7 to 2.5×10^8 TU/animal) (Fig. 3A). SIVmac239 GAG-specific T-cell responses peaked at 16 days after priming

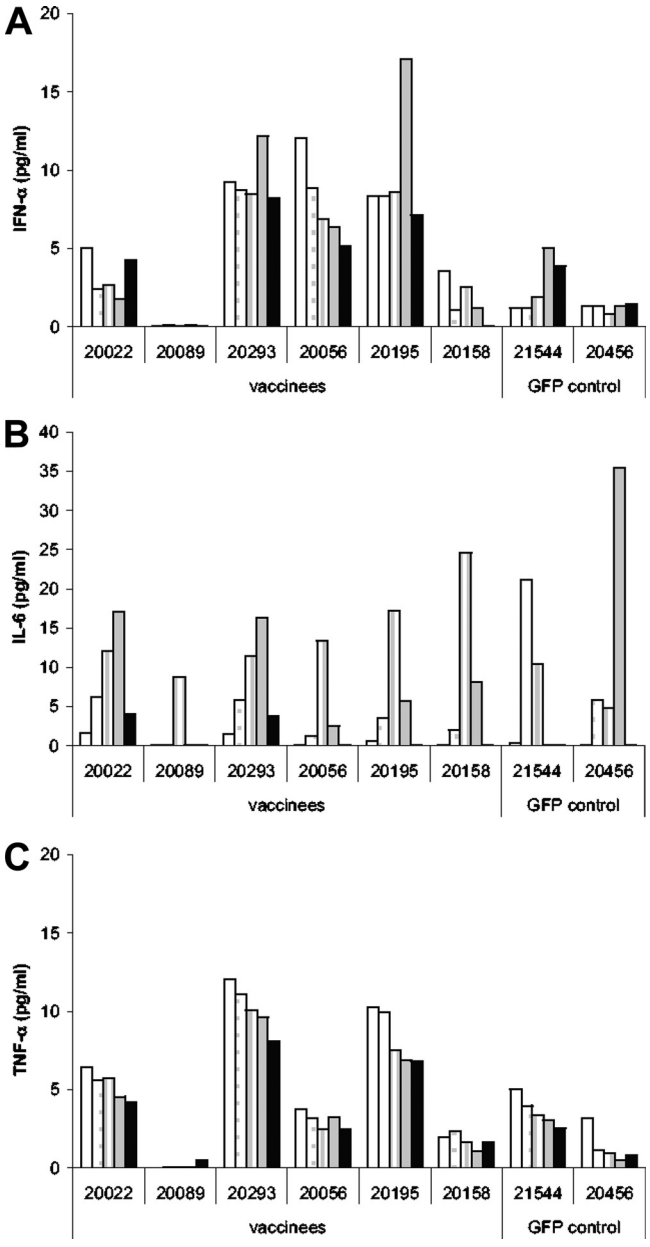
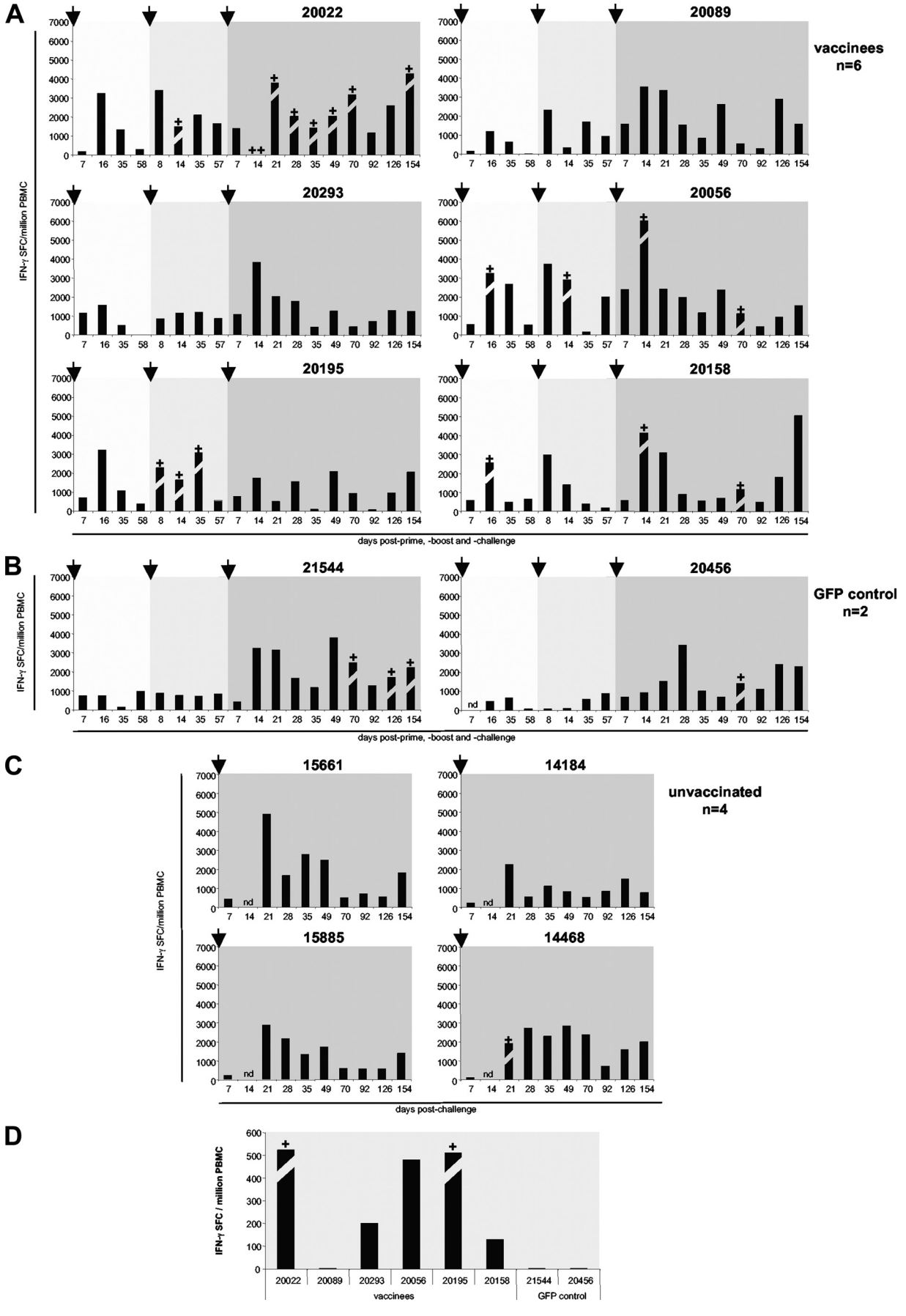


FIG. 2. Subcutaneous injection of lentiviral vector did not result in systemic inflammation. The presence of IFN-α (A), IL-6 (B), and TNF-α (C) in the plasma shortly after subcutaneous injection was measured by ELISA (white, before injection; dots, 1 h after; strips, 6 h after; gray, 1 day after; black, 1 week after). The absence of either significant (IFN-α and TNF-α) or major (IL-6) increases in their level suggested that there was not systemic inflammation induced by the in vivo administration of lentiviral vector particles, even at a high dose (2.5×10^8 TU/animal). These data did not exclude a local inflammation likely triggered by intrinsic pathogen-associated molecular pattern (PAMP) (7, 20, 43).


A
20022
20089
20293
20056
20195
20158
vaccinees
n=6
IFN-γ SFC/million PBMC
days post-prime, -boost and -challenge
B
21544
20456
GFP control
n=2
nd
C
15661
14184
15885
14468
unvaccinated
n=4
days post-challenge
D
IFN-γ SFC / million PBMC
20022
20089
20293
20056
20195
20158
21544
20456
vaccinees
GFP control

(postprime), reaching a high frequency of IFN-γ-secreting cells (up to 3,000 IFN-γ SFC/million PBMC), and returned to pre-immunization levels 2 months after immunization. For some macaques (animals 20056 and 20158), the frequency of specific T cells at the peak of primary responses was so high that spots overlapped, making it impossible to enumerate them precisely. In these cases, the number of IFN-γ SFC/million was largely underestimated, and saturated responses are indicated by the symbol "+" (Fig. 3).

The cellular responses were SIV GAG specific, resulting from the presentation of peptides derived from SIV GAG encoded by the transferred gene rather than from the presentation and cross-presentation of peptides derived from HIV-1 GAG present in the vector particles (since SIV and HIV-1 GAG share sequence similarities), as shown by the low reactivity observed in the two GFP control animals (Fig. 3B) compared to the reactivity observed in the vaccinees (Fig. 3A).

In addition to the robustness of primary responses, these were also found to be multispecific, reacting with an average of three pools of peptides out of eleven (from one for macaque 20089 to six for macaque 20195) (Table 2, section 1, far-right column). In our outbred cohort, we observed that the SIVmac239 GAG specific IFN-γ responses were preferentially directed against two pools within the C-terminal region of GAG covering a part of p27 CA and p9 NC. All six vaccinees mounted a vigorous response against the pool covering SIVmac239 GAG:337-395, and five of six mounted a vigorous response against the pool covering SIVmac239 GAG:385-443 (Table 2, section 1, bottom row). Such concordant immune responses may reflect the simple MHC genetics and the extensive sharing of MHC haplotypes, which characterize Mauritian cynomolgus macaques (57). These two pools of peptides actually contain the GAG GW9 peptide recently described as an immunodominant epitope restricted by Mafa-A*25 and Mafa-A*29, two MHC class IA alleles expressed by 88% of the Mauritian cynomolgus macaques (11).

Animals also developed neutralizing humoral responses against VSV-G serotype Indiana that were dose dependent (Fig. 4A). However, importantly, sera from vaccinated animals did not neutralize vectors pseudotyped with VSV-G New Jersey in vitro (Fig. 4B). These results were in agreement with studies using VSV as a vector (46) or VSV-G-pseudotyped single-cycle SIV (26).

Boost effect of a second injection with lentiviral particles pseudotyped with a non-cross-reactive VSV-G envelope. Since no clear dose response after the prime injection was observed, we decided to boost the animals using an intermediate quantity (1.2×10^8 TU/animal) of TRIP-SIVmac239 GAG particles pseudotyped with VSV-G New Jersey at 11 weeks postprime. This delay between the prime and boost was dictated by the expansion-contraction phases of the vaccine-induced primary T cells responses, assessed by IFN-γ ELISPOT assay.

SIVmac239 GAG-specific T-cell responses were efficiently restimulated by the second injection (Fig. 3A). The magnitude of responses was increased, with kinetics typical of secondary responses, that is, faster onset and longer persistence. IFN-γ-secreting cells were detected as early as 1 week after the second immunization and at up to 2 months and more. The breadth of the cellular responses was not improved (Table 2, section 2). To mimic more closely the processing and trafficking steps that occur in infected cells for antigen presentation but which are bypassed by peptide pulsing, AT-2-inactivated SIVmac239 was also used as antigen. Weak (macaque 20089) to strong (≥500 IFN-γ SFC/million PBMC for macaques 20022, 20195, and 20056) responses were observed (Fig. 3D).

Given the robust and broad cellular immune responses induced by the vaccine, we tested its protective efficacy against SIV infection. Macaques were challenged 11 weeks postboost by intrarectal inoculation of a massive dose of SIVmac251 (500 50% animal infective doses [500 AID_{50}]), 10- to 25-fold more virus than typically used (Table 1 and Fig. 1). Strong anamnestic SIV GAG specific responses were observed in the peripheral blood of immunized animals shortly after challenge (within a week) in contrast to unvaccinated and GFP control animals. These responses peaked earlier and more vigorously (more than 4,000 SIV GAG specific IFN-γ SFC/million PBMC) (Fig. 3A to C). An earlier and higher rebound of total, $CD28^+$ $CD95^-$ naive, and $CD28^+$ $CD95^+$ memory $CD8^+$ T cells was also documented during primary infection in vaccinated animals compared to unvaccinated and TRIP-GFP injected control animals (data not shown). GAG regions mapped after immunizations were recalled by the challenge, and new immunogenic regions were also detected after infection (Table 2, section 3). The diversity of the GAG-specific responses was comparable for vaccinated and unvaccinated or GFP control animals, however (Table 2, section 3).

FIG. 3. A prime/boost lentiviral vector-based vaccination strategy induces robust cellular immunity. The longitudinal follow-up of the SIVmac239 GAG specific T-cell responses was performed at various time points postprime (light gray), postboost (medium gray), and postchallenge (dark gray) by IFN-γ ELISPOT assay after restimulation of whole PBMC with pools of overlapping peptides encompassing SIVmac239 GAG p55. The individual GAG-specific cumulative responses of all six vaccinated animals injected with TRIP-SIVmac239 GAG (low dose, animals 20022 and 20089; medium dose, animals 20293 and 20056; and high dose, animals 20195 and 20158) (A), two control animals immunized with an irrelevant antigen (TRIP-GFP) at a high p24 dose (animals 21544 and 20456) (B), and unvaccinated animals (animals 15661, 14184, 15885, and 14468) (C) are shown. Arrows indicate when the first injection (three doses), the second injection (identical dose), and the SIVmac251 challenge were performed. Aldrithiol AT-2 inactivated SIVmac239 was also used to restimulate GAG-specific $CD4^+$ and $CD8^+$ T cells 2 weeks postboost in a whole PBMC IFN-γ ELISPOT assay. Background after coculture with the control microvesicles was subtracted (D). When the frequency of specific T cells was high and spots overlapped, the number of IFN-γ SFC/million was underestimated to 1,400 before the background was subtracted. Indeed, the saturation curve of the ELISPOT reader was calculated by using serial dilutions of PBMC, and the maximum number of spots enumerated per well appeared to be 280 spots, corresponding to 1,400 spots/million PBMC when 200,000 cells were cultured. The symbol "+" was added at the top of the histogram when saturation was reached for at least one pool of peptides. At 2 weeks postchallenge, it was not possible to quantify the number of spots in the control medium without peptide wells and thus to calculate the cumulative response for animal 20022 (noted as "++"). nd, Not determined.

TABLE 2. Lentiviral vectors expressing SIV GAG induced broad T-cell responses[a]

Section and treatment group	Animal	Lentiviral vectors expressing SIV GAG induced broad T-cell responses[b]											Total
		p17 MA:1-132			p27 CA:133-380					p9 NC and p6:381-511			
		GAG: 1-59	GAG: 49-107	GAG: 97-155	GAG: 145-203	GAG: 193-251	GAG: 241-293	GAG: 289-347	GAG: 337-395	GAG: 385-443	GAG: 433-491	GAG: 481-511	
Two wk postprime													
Vaccinees	20022	140	113	90	160	17	100	63	*743*	*613*	**1,190**	23	3/11
	20089	158	133	11	138	0	0	1	**433**	185	70	68	1/11
	20293	28	**542**	27	180	0	10	45	388	*265*	37	62	3/11
	20056	100	35	102	*405*	77	60	15	**1,280**	*843*	*325*	0	4/11
	20195	*255*	**1,060**	32	*245*	28	*203*	95	*690*	*543*	31	24	6/11
	20058	92	150	165	*297*	55	47	*218*	**900**	*503*	128	8	4/11
GFP control	21544	59	192	60	120	38	59	41	21	42	43	69	0/11
	20456	55	100	0	**325**	0	0	0	0	0	0	0	1/11
Total		1/8	2/8	0/8	4/8	0/8	1/8	1/8	6/8	5/8	2/8	0/8	
One wk postboost													
Vaccinees	20022	173	153	*207*	132	93	123	85	*623*	*402*	**1,347**	65	4/11
	20089	*321*	*291*	150	198	59	176	49	**449**	*434*	135	72	4/11
	20293	167	41	0	3	49	0	27	140	**302**	45	79	1/11
	20056	168	82	88	160	*222*	125	109	**1,275**	*1,150*	*308*	62	4/11
	20195	84	*430*	2	21	11	4	9	*432*	*432*	*432*	432	5/11
	20058	197	70	24	134	*279*	30	88	**1,029**	*909*	177	46	3/11
GFP control	21544	140	**249**	0	199	98	29	0	5	100	49	23	1/11
	20456	0	38	0	20	0	0	0	0	0	17	0	0/11
Total		1/8	3/8	1/8	0/8	2/8	0/8	0/8	5/8	6/8	3/8	1/8	
Three wk postchallenege													
Vaccinees	20022	190	85	78	82	182	103	55	*850*	*873*	**1,197**	92	3/11
	20089	**735**	161	93	52	*523*	*322*	106	*513*	*550*	187	110	5/11
	20293	0	*495*	40	12	0	40	*240*	*510*	**520**	40	128	4/11
	20056	60	0	4	*270*	0	117	33	*602*	**788**	*530*	9	4/11
	20195	99	34	0	16	0	3	172	58	14	60	62	0/11
	20058	142	135	4	*447*	65	58	192	*586*	**658**	*633*	178	4/11
GFP control	21544	147	178	142	*647*	70	118	147	*513*	**807**	198	192	3/11
	20456	13	*252*	18	*205*	105	119	*272*	*217*	152	123	59	4/11
Naive	15661	*288*	**911**	*408*	*228*	0	*513*	161	*906*	*893*	*503*	102	8/11
	14184	170	173	78	*268*	33	88	**403**	*312*	*288*	137	*292*	5/11
	15885	148	136	159	*251*	188	**598**	*326*	*491*	*331*	*229*	12	6/11
	14468	0	46	122	72	29	0	153	37	*320*	**1,033**	92	2/11
Total		2/12	3/12	1/12	7/12	1/12	3/12	4/12	10/12	10/12	6/12	1/12	

[a] The diversity and the relative contributions of the proteins encoded by GAG (matrix [MA], capsid [CA], nucleocapsid [NC], and p6) to the vaccine-induced, virus-induced, and virus-recalled GAG-specific T-cell responses were studied by IFN-γ ELISPOT assay at the peak of the primary responses (2 weeks postprime), a week after the boost, and during the acute phase of infection (3 weeks postchallenge) using 11 pools of 5 to 12 peptides as shown in the column subheadings. The first two columns indicate the animal identification and treatment group.

[b] The numbers correspond to IFN-γ SFC/million PBMC. The underscore indicates saturated ELISPOT wells. The italic entries indicate positive responses (>200 IFN-γ SFC/million PBMC), and the boldface entries indicate the strongest response in an individual animal. The "Total" column shows the numbers of pools of peptides recognized by each animal, whereas the "Total" rows show the numbers of animals of the cohort that mounted a response against each individual pool of peptides.

Protection against a SIVmac251 challenge. Although viral challenge led to infection in all animals (Fig. 5A and B), immunization conferred protection against viral replication and depletion of the $CD28^+$ $CD95^+$ memory $CD4^+$ T cells during the acute phase. TRIP-GFP-injected control animals showed a course of infection very comparable to that of unvaccinated macaques and were therefore gathered as a single group (Fig. 5A). In the plasma of these naive and GFP control animals, the peak of viral replication was high with a geometric mean of 3.19×10^6 viral RNA copies/ml. Viral loads then decreased in all six unvaccinated and GFP control animals to reach low to moderate set-point plasma viral RNA levels (days 70 to 154) with a geometric mean of 4.65×10^4 viral RNA copies/ml (Fig. 5A). In contrast, viremia at the peak of acute infection of all six

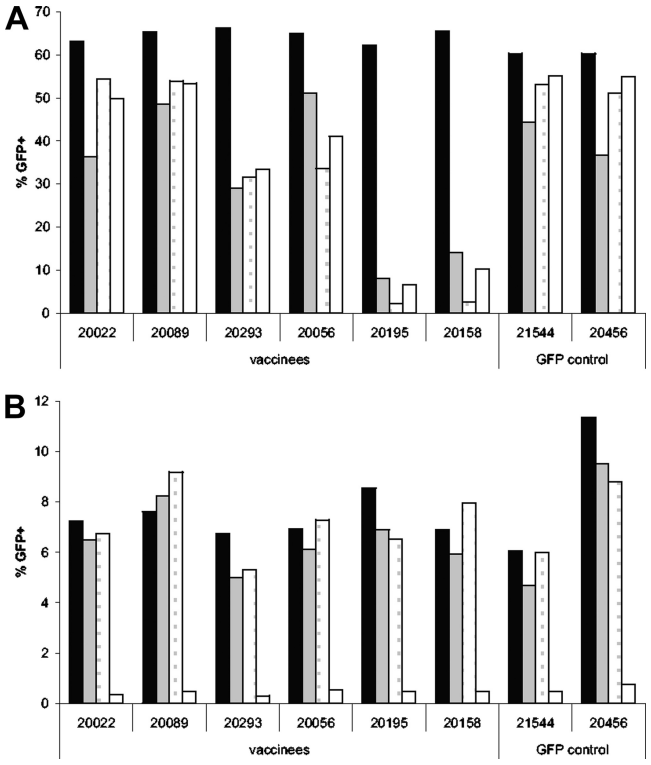


FIG. 4. Injected animals develop humoral responses to VSV-G used to pseudotype the vector particles. The presence of neutralizing antibody against the envelope used for pseudotyping was measured with an in vitro transduction assay. P4 cells (HeLa derived) were cultured in the presence of lentiviral vectors encoding GFP-pseudotyped with VSV-G Indiana (A) or VSV-G New Jersey (B) preincubated with plasma diluted at 1:20 from immunized animals collected at various time points (black, before priming; gray, 2 weeks postprime; dots, 8 weeks postprime; and white, 2 weeks postboost). The transduction efficacy was assessed by flow cytometry. In the absence of plasma and at the dose of vector used, 61 and 23% of P4 cells were GFP^+ after transduction with lentiviral vectors encoding GFP pseudotyped with VSV-G Indiana and New Jersey, respectively.

immunized animals were lower than in naive and GFP control animals by at least 2 orders of magnitude with a geometric mean of 1.52×10^4 viral RNA copies/ml (Fig. 5B and C). From the six vaccinated macaques, four suppressed peak viremia by more than 2 $\log_{10}$ fold (macaques 20022, 20293, 20158, and 20195), two suppressed peak viremia by more than 3 $\log_{10}$ fold (macaques 20293 and 20158), and one suppressed peak viremia by more than 4 $\log_{10}$ fold (macaque 20195) (Fig. 5E). After resolution of the peak viremia, the viral loads decreased (Fig. 5B) and remained persistently below those of unvaccinated and control animals, but these differences were not statistically significant by day 49 postinfection (Fig. 5C). However, when the cumulative replications during the first 154 days of infection (expressed as the area under the curve of viral load as a function of time) were compared, the benefit provided by vaccination was statistically significant (data not shown).

We also monitored the evolution of $CD4^+$ T cells in the peripheral blood during the course of infection, and more particularly the subset of $CD28^+$ $CD95^+$ memory $CD4^+$ T cells, because their depletion correlates with plasma viral loads (28) and their preservation during acute and chronic SIV infection predicts long-term survival of vaccinated monkeys better than set-point viral load levels (30, 34). During acute infection, there was a rapid and profound decline of the $CD28^+$ $CD95^+$ $CD4^+$ T cells in the peripheral blood of the unvaccinated and GFP control animals (Fig. 6A). $CD28^+$ $CD95^+$ $CD4^+$ T-cell counts remained low with signs of gradual depletion for half of them (macaques 21544, 14184, and 20456), whereas depletion was transient and followed by a return to baseline for the other half (macaques 15661, 15885, and 14468). These two subgroups further demonstrated moderate and low post-acute-phase viremia correspondingly (Fig. 5A) and were therefore classified as progressor (macaques 14184, 21544, and 20456) and slow progressor control animals (macaques 15661, 15885, and 14468) (Fig. 5D and 6D). Vaccinated animals showed full preservation or only low depletion of their $CD28^+$ $CD95^+$ $CD4^+$ T cells during peak viremia, and all of them rapidly recovered their $CD28^+$ $CD95^+$ $CD4^+$ T lymphocytes, except macaque 20089 (Fig. 6B). Thus, the vaccine conferred protection against the severe depletion of the $CD28^+$ $CD95^+$ memory $CD4^+$ T-cell compartment (Fig. 6C and E). This preservation also applied to total $CD4^+$ T cells (Fig. 6F). Importantly, viremia of vaccinated animals at late time points was reduced by around a 1 $\log_{10}$ fold factor compared to progressor control animals (Fig. 5D), whereas post-acute-phase viremia and $CD28^+$ $CD95^+$ $CD4^+$ T-cell counts were strikingly similar between vaccinees and slow progressor control animals (Fig. 6D).

MHC haplotype. We characterized the individual MHC haplotypes (Fig. 7). All MHC haplotypes observed in our cohort ($n = 12$) were consistent with those previously described for feral Mauritian cynomolgus macaques and breeding colonies (37, 57). Animals carried one of the complete seven common haplotypes, with H1, H2, and H3 being the most common, or simple recombinants thereof and none of them was homozygous. None matched for an entire MHC class I and II haplotype, however, some pairs of macaques shared an identical MHC class I or class II genetics. Some associations between the MHC class I and class II genotypes and the control of SIV/SHIV replication have been recently identified in cynomolgus macaques from Mauritius (19, 38). It should be noted, however, that studies with larger cohorts are required to confirm the effects of some MHC haplotypes on the susceptibility or resistance to SIV/SHIV infection reported. It was shown that MHC class IB and class II H2 and H5 haplotypes were associated with a higher susceptibility to SHIV89.6P infection, whereas MHC class IB haplotypes H3 and H6 were associated with resistance to SHIV89.6P infection (in both the acute and the chronic phases) (19). In addition, not only MHC class IB H6 haplotype but also class II haplotype H6 was reported to be associated with lower chronic viremia after SIVmac251 infection (38). It appeared that without prior selection, our macaques were distributed quite evenly among experimental groups, in particular, there was no over-representation of the "favorable" H3 or H6 haplotypes in the vaccine arm and of the "disadvantageous" H2 or H5 haplotypes in the control arm, thus limiting a major bias of MHC on the vaccination and challenge outcomes.

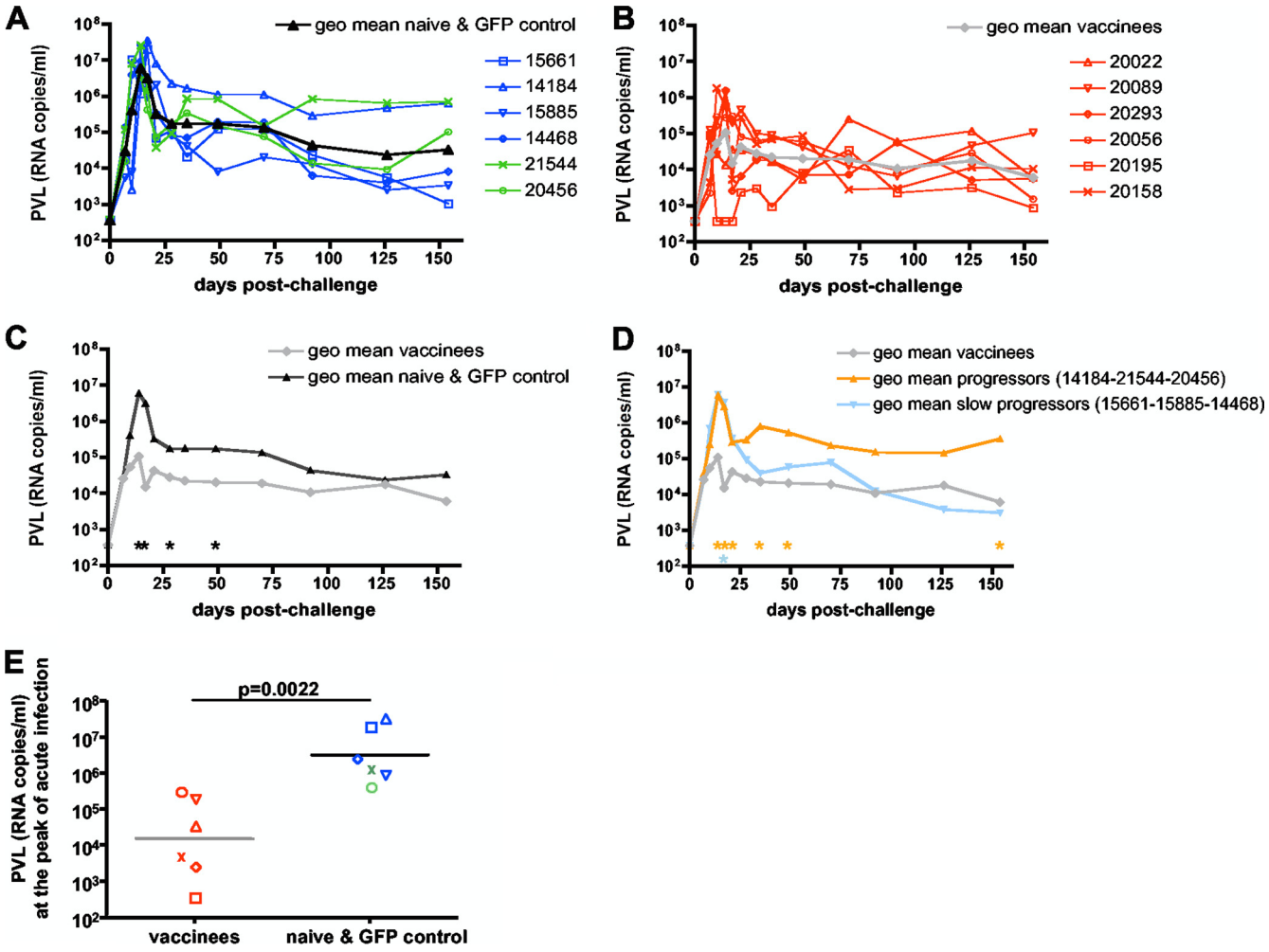


FIG. 5. Vaccinated macaques have an improved control of viremia compared to unvaccinated and GFP control animals. Plasma viral loads were monitored for 5 months postchallenge, twice a week during the first 3 weeks, then once a week during the next 3 weeks and finally once a month. Viremia of unvaccinated (panel A in blue), GFP control (panel A in green), and vaccinated animals (panel B in red), as well as the geometric mean for the naive and GFP control group (in black) versus the vaccinated group (in gray) (A to C) are shown. The geometric mean of viral replication levels was lower in the vaccinated group at all time points tested (C). An average 2 $\log_{10}$ fold reduction of viremia was observed at the peak of acute infection (E). The geometric mean viremia of the vaccinated animals (in gray) was also compared to that of progressor animals (14184, 21544, and 20456) in orange and of slow progressor animals (15661, 15885, and 14468) in light blue (D). Post-acute-phase viremia levels were lower in vaccinated animals than in progressor animals. *, $P < 0.05$.

DISCUSSION

This pilot study highlights the potential of nonreplicative HIV-1-derived gene transfer vectors as a candidate AIDS vaccine. A strong and diverse cellular immunity was elicited in macaques (Fig. 3), which resulted in a significant protection against early viral replication after a massive SIVmac251 challenge (Fig. 5). With the exception of one low vaccine responder out of six immunized macaques, the animals showed a strong reduction of viremia during acute infection (from 1 to 4 $\log_{10}$ peak reduction, with a geometric mean of >2 $\log_{10}$), with a concomitant preservation of the $CD28^+$ $CD95^+$ memory $CD4^+$ T cells (Fig. 6). A long-term follow-up is needed to determine whether vaccinees can control viral replication durably, maintain a large pool of $CD28^+$ $CD95^+$ memory $CD4^+$ T cells and ultimately survive longer than control animals (34, 42).

A direct quantitative comparison of the level of protection achieved by this novel vaccination strategy with other T-cell-based approaches is made difficult by the use of monkeys from different species or origins between studies. Although most trials are conducted using the well-characterized Indian rhesus macaque model, these animals are not easily available in Europe, and our study was carried out with Mauritian cynomolgus macaques. Cynomolgus macaques are used for preclinical vaccine evaluation. Various vector strategies have been tested, including DNA, recombinant Semliki Forest and modified recombinant vaccinia virus strain Ankara (MVA) (33, 39, 40). It is not known whether cynomolgus macaques are easier or not to protect in a vaccination protocol against simian AIDS than Indian rhesus monkeys. What is known, however, is that, in Mauritian cynomolgus macaques as in rhesus macaques, live attenuated SIV are protective (4) and some MHC haplotypes

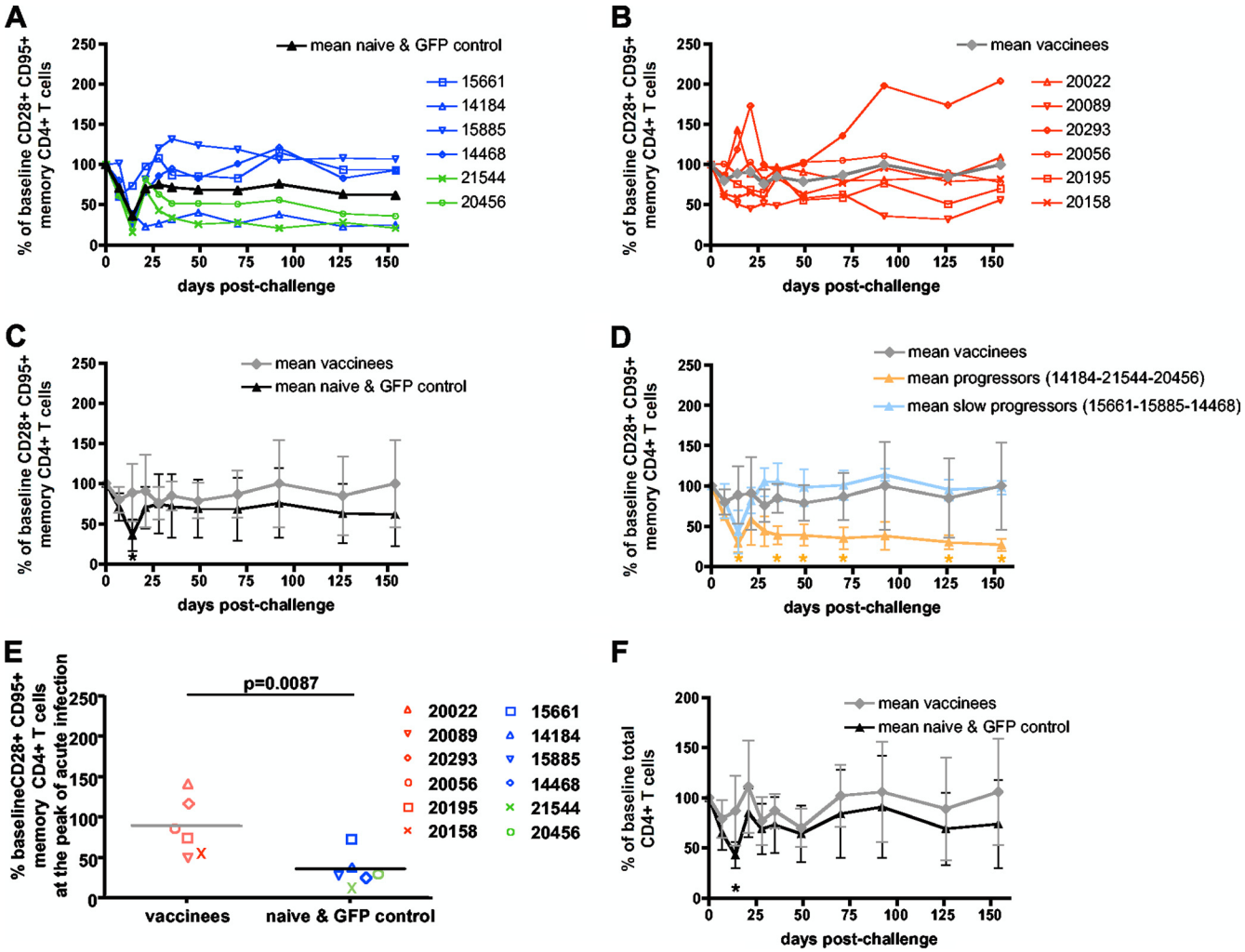


FIG. 6. The total and CD28$^+$ CD95$^+$ memory CD4$^+$ T-cell compartments are well preserved in vaccinated macaques. Changes in the numbers of CD28$^+$ CD95$^+$ memory CD4$^+$ T cells in the peripheral blood were monitored for 5 months postchallenge. The percentages of baseline CD28$^+$ CD95$^+$ CD4$^+$ T cells of unvaccinated (panel A in blue), GFP control (panel A in green), and vaccinated animals (panel B in red), as well as the mean for the naive and GFP control group (in black) versus the vaccinated group (in gray) (A to C), are shown. Vaccinated animals showed a full preservation of their CD28$^+$ CD95$^+$ CD4$^+$ T-cell compartment during acute infection in contrast to naive and GFP control animals (C) and no gradual depletion in the chronic phase in contrast to progressor animals (14184, 21544, and 20456) in orange (D). *, $P < 0.05$. CD28$^+$ CD95$^+$ memory CD4$^+$ T cells for all animals are compared at the peak of acute infection (E). The dynamics of total CD4$^+$ T cells in vaccinees (in gray) also differ from those of control animals (in black) (F). *, $P < 0.05$.

can influence the resistance or susceptibility to SIV/SHIV infection (19, 38). Although the MHC phenotype was not a controlled variable in our study, it has probably not strongly biased the protective effect induced by our lentiviral vector-based candidate vaccine, as suggested by our retrospective individual MHC haplotype analysis (Fig. 7). Anyway, a quantitative assessment of the different levels of protection achieved by different candidate vaccines would require a side-by-side comparative study of the various strategies in the same SIV/macaque model.

Nevertheless, the lentiviral vector-based vaccine that we developed provides at least an early viral replication control similar to that obtained in macaque preclinical trials using the best T-cell-based strategies described thus far. In one study, a heterologous DNA prime/rAd5 boost expressing GAG afforded a 7- and 15-fold reduction of viremia at the peak of infection and at early set-point, respectively, in Indian rhesus macaques after SIVmac239 challenge. However, the control of viral replication was observed only in Mamu-A*01$^+$ monkeys, an allele known to be associated with lower viral loads, and was short-lived (13, 35). A multivalent DNA prime/rAd5 boost (encoding GAG, TAT, REV, and NEF) was also tested in Mamu-A*01$^+$ monkeys. It was more efficient than the monovalent GAG DNA prime/Ad5 boost vaccine and conferred nearly 1.5 $\log_{10}$ reduction of both peak and chronic phase viremia (up to 1 year follow-up) after repeated low-dose SIVmac239 challenge (56). In a third study, Watkins and coworkers assessed the protective efficacy of a ADN prime/rAd5 boost vaccine regimen (encoding VIF, VPR, and VPX in addition to GAG, TAT, REV, and NEF) against a repeated mucosal heterologous SIVsmE660 challenge after excluding animals carrying the protective alleles Mamu-A*01, Mamu-

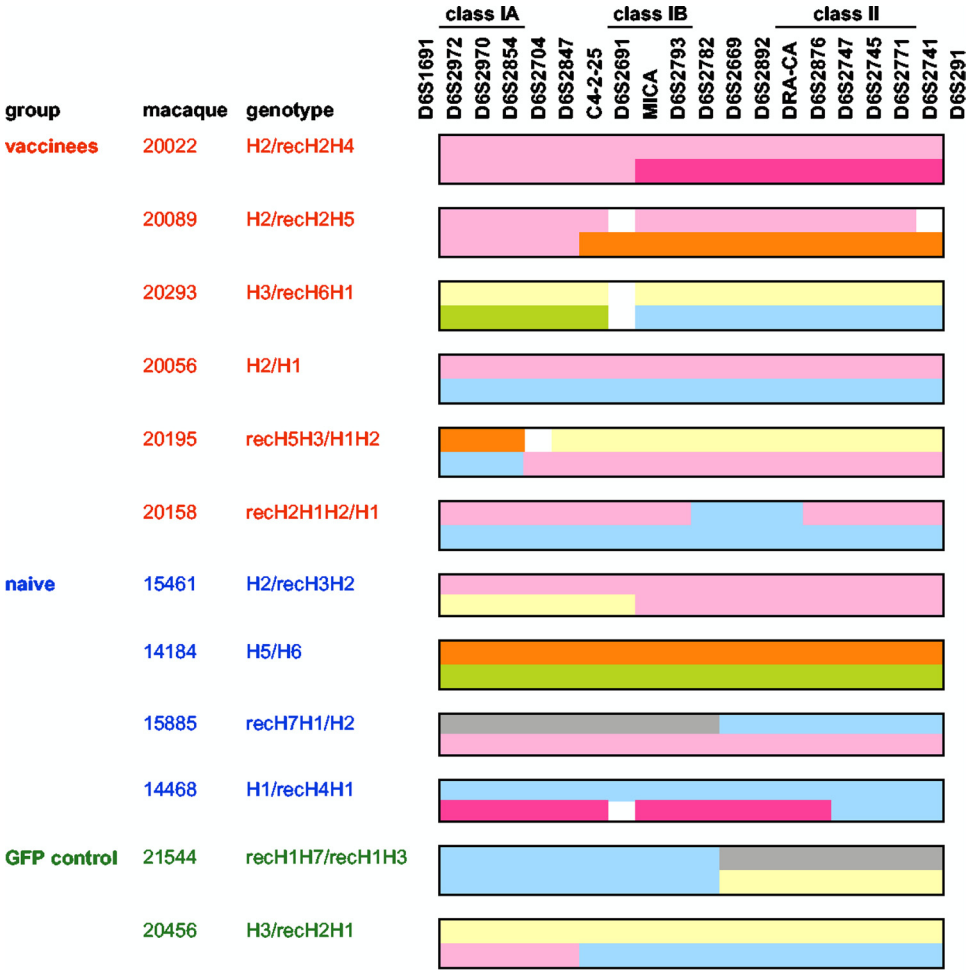


FIG. 7. Individual MHC haplotypes of the animals included in the cohort. MHC haplotypes (H1 to H7) were determined by microsatellite analysis. Intact and recombinant haplotypes are indicated for each chromosome of each animal and are color-coded. White boxes indicate variant microsatellite allele sizes relative to the expected haplotype (these rare variants generally differ by the addition or loss of a single repeat unit).

B*08, and Mamu-B*17 (55). Viremia in vaccinees were reduced both at the peak (1.9 $\log_{10}$ reduction) and at set-point (2.6 $\log_{10}$ reduction from days 42 to 140 postinfection). A heterologous rAd26 prime/rAd5 boost vaccine regimen expressing only GAG was also reported to afford durable immune control of viral replication (up to 1 year follow-up, with 1.4 and 2.4 $\log_{10}$ reduction of peak and set-point viral loads, respectively) after infection with SIVmac251 of Mamu-A*01$^-$ Indian rhesus macaques (31). Finally, a long-term control of viremia after SIVmac239 challenge was observed after immunization of Indian rhesus macaques with a single cycle SIV (12- and 52-fold lower viremia at the peak and in the chronic phase, respectively) (26).

In any case, none of these approaches, including ours, reached the stable and strong levels of protection afforded by replicative attenuated SIV against homologous challenge (32, 45, 49). However, the results we obtained in this first proof-of-concept study make a strong case for the potential of a lentiviral vector-based vaccination against AIDS and suggest that the strategy we developed will achieve increased levels of protection upon further optimization. First, the benefits of vaccination we observed relied solely on responses mounted against a nonoptimized and single antigen (GAG). A sustained control of replication can be anticipated from an optimized antigen design, by increasing antigen expression and immunogenicity by codon optimization (16, 60; our unpublished results) and by increasing the diversity of the cellular responses by fusing other SIV antigens with GAG (23, 56).

The facilitation of HIV infection that was reported during the adenoviral vector-based STEP trial (8, 36) can be excluded using a lentiviral vector-based vaccine. First, vector preimmunity is almost absent in humans. Indeed, VSV infection prevalence is very low in humans, leading to very rare anti-VSV-G envelope preimmunity. Second, VSV-G pseudotyped lentiviral vectors do not exhibit a tropism bias toward mucosal epithelial cells. Third, the lentiviral vector doses injected in vivo are very low compared to adenoviral vectors (ca. 10^8 versus 10^{11} vector particles, respectively, in macaques). This precludes the inflammation of the genital mucosa after in vivo injection of a lentiviral vector-based vaccine.

Other vaccination trials in macaques using larger cohorts, with an optimized antigen and vector design, will provide a full evaluation of the vaccination potential of lentiviral vectors against simian AIDS. A particular emphasis on the

nature and quality of the vaccine-induced cellular immunity will be helpful for the understanding of the mechanisms of protection. An optimized version of this vaccination strategy, with complete fulfillment of both efficacy and safety requirements, will then enter therapeutic vaccination clinical trials in humans.

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P.C. is a founder of TheraVectys, and K.M. is an employee of and shareholder in TheraVectys, a company whose goal is the development of an AIDS therapeutic vaccine.

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